A Soluble Form of the *Tomato spotted wilt virus* (TSWV) Glycoprotein G_N (G_N-S) Inhibits Transmission of TSWV by *Frankliniella occidentalis*

A. E. Whitfield, N. K. K. Kumar, D. Rotenberg, D. E. Ullman, E. A. Wyman, C. Zietlow, D. K. Willis, and T. L. German

First, third, fifth, and eighth authors: Department of Entomology, University of Wisconsin, Madison 53706; second, fourth, and sixth authors: Department of Entomology, University of California, Davis 95616; seventh author: Department of Plant Pathology, University of Wisconsin and U.S. Department of Agriculture, Vegetable Crops Research Unit, Madison, WI 53706.
Current address of first and third authors: Department of Plant Pathology, Kansas State University, Manhattan 66506.

ABSTRACT

Whitfield, A. E., Kumar, N. K. K., Rotenberg, D., Ullman, D. E., Wyman, E. A., Zietlow, C., Willis, D. K., and German, T. L. 2008. A soluble form of the *Tomato spotted wilt virus* (TSWV) glycoprotein G_N (G_N-S) inhibits transmission of TSWV by *Frankliniella occidentalis*. Phytopathology 98:45-50.

Tomato spotted wilt virus (TSWV) is an economically important virus that is transmitted in a persistent propagative manner by its thrips vector, *Frankliniella occidentalis*. Previously, we found that a soluble form of the envelope glycoprotein G_N (G_N-S) specifically bound thrips midguts and reduced the amount of detectable virus inside midgut tissues. The aim of this research was to (i) determine if G_N-S alters TSWV transmission by thrips and, if so, (ii) determine the duration of this effect. In one study, insects were given an acquisition access period (AAP) with G_N-S mixed with purified virus and individual insects were assayed for transmission.

We found that G_N-S reduced the percent of transmitting adults by eight-fold. In a second study, thrips were given an AAP on G_N-S protein and then placed on TSWV-infected plant material. Individual insects were assayed for transmission over three time intervals of 2 to 3, 4 to 5, and 6 to 7 days post-adult eclosion. We observed a significant reduction in virus transmission that persisted to the same degree throughout the time course. Real-time reverse transcription polymerase chain reaction analysis of virus titer in individual insects revealed that the proportion of thrips infected with virus was reduced threefold when insects were preexposed to the G_N-S protein as compared to no exposure to protein, and nontransmitters were not infected with virus. These results demonstrate that thrips transmission of a tospovirus can be reduced by exogenous viral glycoprotein.

Additional keywords: Bunyaviridae, plant virology, *Thysanoptera*, virus entry, virus–vector interactions, western flower thrips.

The family *Bunyaviridae* is composed of enveloped, negative-strand RNA viruses that infect a large number of plant, arthropod, and mammalian species. *Tomato spotted wilt virus* (TSWV) is the type member of the genus *Tospovirus* in the family *Bunyaviridae*. The TSWV virion is composed of three RNA segments (large, medium, and small) that are enclosed in a host-derived membrane. The two glycoproteins, G_N and G_C, are encoded on the medium RNA segment and are translated as a polyprotein that is proteolytically processed to yield the two mature proteins. G_N and G_C decorate the surface of the virion and are approximately 58 and 95 kDa, respectively (18,29) and bunyavirus glycoproteins have been shown to play an essential role in virus infection of host cells. Plant hosts of TSWV include food, fiber, and ornamental crops encompassing hundreds of plant species (4). In addition to the large plant host range, TSWV also replicates in its insect vector, thrips.

Thrips are members of the insect order *Thysanoptera*, and *Frankliniella occidentalis* (Pergande) is one of the most economically important agronomic pests within this order because it is both a direct pest of crops and a vector of plant viruses. *F. occidentalis* is polyphagous, feeding on a wide array of plant species and plant organs. It is the most efficient vector of tospoviruses, transmitting 5 of the 14 currently described *Tospovirus* species (33). TSWV is transmitted in a persistent propagative fashion and is transstadially passed in its insect vector. The thrips-tospovirus relationship is unique because adult thrips can only transmit TSWV if acquisition occurs in the larval stages (30). Adult thrips that feed on infected plants are unable to transmit virus even if they are allowed lengthy feeding periods on tospovirus-infected plants (30).

Understanding the process of virus transmission requires a detailed consideration of the multiple points of interaction between virus and vector that govern entrance, replication, movement within, and exit from the vector. The TSWV infection cycle in the insect can be divided into several steps and the virus must traverse at least six membrane barriers for transmission to occur. Insects feed on infected plants and TSWV enters the midgut lumen. Virions must move across the midgut apical membrane of the brush border and this is potentially mediated via an interaction with the TSWV glycoproteins and a midgut receptor. The virus likely binds a receptor on the surface of the midgut epithelial cells and enters via the endocytotic pathway (10,13). Once the virus enters the insect midgut cells, it has been acquired by the larval thrips. The virus replicates and spreads to adjacent midgut cells eventually moving into the muscle cells surrounding the midgut (31). For transmission to occur, the virus must move from the gut tissues to the primary salivary glands. TSWV replicates in the salivary glands and virions exit the salivary gland across the apical membrane and flow with the salivary secretions into the plant during thrips feeding (33). Taken together, these steps comprise the insect infection events that culminate in the inoculation of a plant host.

Multiple studies of TSWV-thrips interactions and of other bunyaviruses provide evidence that glycoproteins play an

important role in the infection of insect vectors and animal cells. The two glycoproteins decorate the surface of the virion, and therefore are probably the first viral components that interact with molecules in the thrips midgut. Isolates of TSWV that are serially, mechanically passed to plants generate mutations and deletions in the glycoprotein open reading frame (ORF), rendering these viruses nontransmissable by thrips (20,25). Work by Sin et al. (27) showed that the TSWV glycoproteins are necessary for virus infection of *F. occidentalis* and that a single point mutation in the glycoprotein ORF abolished transmission of the virus by insects. However, these alterations in the glycoprotein ORF do not compromise the ability of the virus to infect plants. Furthermore, G_N and G_C anti-idiotypic antibodies specifically label the midgut when they are incubated with dissected thrips (2). For other members of the *Bunyaviridae*, the glycoproteins are important in virus entry (10,16,22,28). Antibodies to G_C (14,23) and/or G_N (15,26) neutralize virus infection of animal cells, and reassortment studies with hantaviruses and orthobunyaviruses have shown that virulence maps to the medium RNA segment that encodes G_N and G_C (6,8,12).

Characterization of the role of TSWV G_N in the virus infection process in thrips is underway. In previous work, we demonstrated that a soluble form of the membrane surface glycoprotein (G_N-S) of TSWV retained many characteristics of the wildtype G_N protein; most importantly, G_N-S bound larval thrips midguts in an in vivo binding assay (32). We found that this binding was specific because the TSWV nucleocapsid (N) protein and another viral attachment protein, human cytomegalovirus glycoprotein gB, did not bind larval thrips midguts. In TSWV-acquisition inhibition assays, we documented significant reductions in the amount of virus in midgut cells of thrips fed concomitantly on purified TSWV and G_N-S compared to thrips fed TSWV alone. Our findings that G_N-S bound to larval thrips guts and decreased TSWV acquisition provide evidence that G_N may serve as a viral ligand that mediates attachment of TSWV to receptors displayed on the epithelial cells of the thrips midgut.

Based on our understanding of the TSWV transmission process in thrips and our previous findings with G_N-S, we hypothesized that an initial reduction in TSWV acquisition reduces the proportion of viruliferous thrips. If such a phenomenon does not occur, it could indicate that the virus successfully crosses the midgut barrier in the presence of G_N-S (acquisition), proceeds to replicate and infect salivary glands, thus leading to a transmission event. The objectives of this study were to determine if G_N-S, fed to thrips concomitantly with purified virus or in succession with TSWV-infected plants, alters transmission by thrips and if so, determine the duration of this effect. Here we report that G_N-S significantly reduced the proportion of transmitting adults and this phenomenon persisted over time. These findings warrant further research to develop strategies for TSWV-control based on the G_N-S-midgut interaction.

MATERIALS AND METHODS

F. occidentalis **cultures.** A colony of *F. occidentalis* was maintained on green bean pods (*Phaseolus vulgaris*) as previously described (30). To generate first instar larvae for transmission experiments, beans were incubated with adult thrips for 3 days to allow the insects to oviposit. Thrips were removed and the beans were incubated at 23°C for 24 h. Larval thrips (0 to 24 h old) were harvested and pooled. Aliquots of the same thrips cohort were transferred to feeding chambers for transmission experiments.

TSWV purification. TSWV (isolate TSWV-MT2) was maintained by thrips transmission as described (30,31), and virus was mechanically transferred only one time after thrips transmission to maintain thrips transmissibility of the isolate. TSWV-infected *Datura stramonium* leaves used for virus purification were harvested 2 weeks postinoculation and TSWV virions were isolated by using a modified and shortened version of the Gonsalves and Truijillo (7) procedure. The triturated and filtered homogenate was centrifuged at $8,000 \times g$ for 15 min. The supernatant was removed, 50 ml of 10 mM of sodium sulfite was added, and pellets were suspended by stirring on ice at 4°C for 95 min. The virus was centrifuged at 4°C for 20 min at $8,600 \times g$ and the supernatant collected. The supernatant was centrifuged for 39 min at $88,000 \times g$. The supernatant was removed and the pellet was suspended in 500 μl sodium sulfite. Before the virus was presented to thrips, protease inhibitors (1 μg/ml each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin) were added to the virus solution. Virus viability was tested by mechanically inoculating *Nicotiana glutinosa*, a local-lesion host.

Concomitant feeding experiments with G_N-S and TSWV. To assay G_N-S for the ability to inhibit TSWV transmission, first instar larval thrips were given an AAP on solutions containing purified TSWV or purified TSWV combined with G_N-S (experiment 1). The G_N-S protein was produced and isolated as previously described (32). Thrips were fed in cylindrical 25-mm-diameter containers similar to the method described by Hunter et al. (11). Ends of the tubes were sealed with a thin layer of parafilm which allowed the insects to feed. The feeding solutions were (i) TF buffer (PBS, 10% glycerol, 0.01% Chicago sky blue, and 5 mg/ml of BSA); (ii) G_N-S (0.1 nM) + TSWV in TF buffer; or (iii) TSWV in TF buffer. Insects were fed 0.1 nM of G_N-S protein because this concentration was shown to bind larval thrips midguts and inhibit TSWV acquisition (32). Purified virus solutions were diluted to the same concentration and we used the methods described in Whitfield et al. (32) to manipulate the thrips. One hundred microliters of feeding solution was sandwiched between two layers of parafilm. Larval thrips were allowed to feed on the solutions for 16 h and then transferred to beans to be reared to adulthood. Thirteen days after the AAP, the adult insects were moved to *D. stramonium* leaf disks held in 1.5-ml microfuge tubes. After allowing the thrips to feed for 6 days, the leaf disks were moved to 24-well plates (Falcon) with 1 ml of deionized water per well. Leaf disks were incubated at room temperature for 3 days, ground in a plant leaf grinder, and assayed for virus infection using the QTA Tospo ELISA kit (Agdia, Ekhart, IN). The Fisher exact probability test (1) was used to test the null hypothesis that treatment (buffer, G_N-S + TSWV, or TSWV alone) and outcome (infected or not infected leaf disk) are independent. A two-way contingency table of frequency data for each treatment and outcome status was prepared. The Fisher exact statistic was calculated using the SAS (SAS Institute Inc., Cary, NC) procedure PROC FREQ with the subcommand EXACT FISHER. The two-sided *P* value was used to test the null hypothesis of independence (i.e., no apparent relationship between feeding treatment and infection status (+/–) of the leaf disk).

Sequential feeding experiments with G_N-S and TSWV. We conducted experiments to determine if feeding insects G_N-S prior to AAP on infected plant material (designated G_N-S/TSWV) would reduce the proportion of viruliferous thrips (experiment 2). Young thrips were given a 2 h AAP on G_N-S (0.1 nM in TF buffer) or TF buffer as described for in vivo binding assays in Whitfield et al. (32). Thrips were then placed on a bouquet of TSWV-infected *D. stramonium* leaves for 3 h. Insects were transferred to bean pods and reared to adulthood. After adult emergence, individual thrips were placed on *D. stramonium* leaf disks for 48-h inoculation access periods (IAPs) and disks were replaced three times (2 to 3, 4 to 5, and 6 to 7 days post-adult-eclosion) in order to assess thrips transmission over time. Leaf disks were incubated in water for 4 days and TSWV detected by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Agdia, Elkhart, IN). The proportion of leaf disks infected with TSWV was calculated and expressed as arcsin√(per-

cent infection/100) to normalize data and associated residuals. The experiment was conducted three times and a total of 104 and 107 thrips were given an AAP with the G_N-S/TSWV and buffer/TSWV (TSWV alone) treatments, respectively. Each experiment repeat represented a different group of thrips (three independent replicates per feeding treatment). Analysis of variance (ANOVA) was performed on transformed percent infection to determine (i) if thrips fed G_N-S exhibited reduced transmission as determined by incidence of leaf infection and (ii) if the ability of an insect to transmit virus persisted over time. The statistical model included two main effects (feeding treatment and time after adult eclosion) and the interaction term (treatment–time). The model was tested with the SAS procedure PROC MIXED using the REPEATED statement, with thrips group as the subjects measured over time. The LSMEANS statement was used to calculate least square differences and P values for making comparisons among feeding treatments at each time point.

Real-time RT-PCR analysis of TSWV in thrips. To determine the infection status (incidence and titer of virus) of insects, we quantified virus titer in individual insects that were fed G_N-S protein, followed by an AAP on TSWV-infected *Datura* leaves, and then allowed a 2 to 3 day IAP on *Datura* leaf disks (similar experimental design as experiment 2). Twenty insects from each treatment (G_N-S/TSWV and TSWV alone) were sampled from their respective leaf disks. RNA was extracted from individual insects using the method of Boonham et al. (3). cDNA was made using the iScript cDNA synthesis kit (Bio-Rad), and 15 µl of RNA was used for each 20-µl reaction. Real-time RT-PCR was performed using the iCycler iQ Thermal Cycler with 96 × 0.2 ml reaction module and iCycler iQ software (Bio-Rad). The *F. occidentalis* actin primers described by Boonham et al. (3) were used for amplification of the actin gene (forward primer 5′-GGTAT-CGTCCTGGACTCTGGTG-3′, reverse primer 5′-GGGAAGG-GCGTAACCTTCA-3′). Primers to the TSWV nucleocapsid (N) gene were developed using Beacon Designer 2.0 (Premier Biosoft International, Cambridge). The forward N primer 5′-GCTTCC-CACCCTTTGATTC-3′ and reverse N primer 5′-ATAGCCAAG-ACAACACTGATC-3′ were tested for efficiency and specificity. Because the TSWV N primers amplify the N gene mRNA and TSWV S RNA, herein we refer to the amplicon as TSWV N RNA. We used iQ SYBR Green (Bio-Rad) for all RT-PCR according to manufacturer's specifications. Briefly, reactions were performed in a volume of 20 µl, using iQ SYBR Green Supermix (Bio-Rad), 20 pmol of forward and reverse primers, and the same volume of cDNA for each reaction. Reactions were performed in the iCycler (Bio-Rad) using the 2 step amplification plus melting curve protocol. Threshold values for threshold cycle (C_t) determination were generated automatically by the iCycler iQ software. Lack of variation in PCR products and the absence of primer-dimers were determined from the melt curve profile of the PCR products. TSWV N gene expression (target) was normalized to actin expression (internal reference) to calculate the relative abundance of TSWV N RNA in each insect using the inverse equation in Pfaffl (24): $E_{actin}^{Ct(actin)}/E_N^{Ct(N)}$; where E = PCR efficiency of a primer pair (actin or N). The proportion of TSWV-infected insects was calculated for each treatment. The Fisher exact test was performed using SAS to determine if the proportion of virus-infected insects (incidence) was independent of treatment. The average amount of virus (titer) harbored in TSWV-positive insects was calculated for each treatment, and treatments were compared using the Mann-Whitney test statistic (MINITAB version 13.31, Minitab, Inc., State College, PA) because of the limited sample size after removal of TSWV-negative insects in the G_N-S treatment.

Local lesion assay. G_N-S was mixed with purified TSWV and rub-inoculated onto local lesion plant hosts, *N. glutinosa*, to determine if G_N-S affects virus viability (measured by infectivity of plant host tissue). Virus was purified as described and mixed

with G_N-S in PBS, pH 7.4, or PBS alone. A small amount of celite (0.05 g) was added to act as an abrasive and the protein-virus mix was diluted in 0.01 M sodium sulfite. The solutions were rub-inoculated onto tobacco leaves using a cotton-tipped applicator. Local lesions were counted 3 days postinoculation and the experiment was conducted twice.

RESULTS

G_N-S **reduced TSWV transmission when fed concomitantly with purified TSWV.** When insects were given an acquisition access period (AAP) on purified virus, 56% of the insects transmitted TSWV to *D. stramonium* leaf disks (Fig. 1). Insects given an AAP on TSWV + G_N-S resulted in an eightfold reduction in the proportion of transmitting adults, compared to insects fed on TSWV alone ($P < 0.01$), indicating that G_N-S inhibited TSWV transmission. The proportion of transmitting insects given an AAP on a buffer alone was not significantly different ($P = 0.5$) from insects exposed to the G_N-S + TSWV mixture. Leaf disks were never TSWV-positive when exposed to insects given an AAP on buffer alone, confirming that the insects and test plants were not contaminated with virus. The data presented here indicate that G_N-S inhibition of acquisition was sufficient to greatly reduce the number of insects that transmitted virus. These results are consistent with our previous finding that G_N-S specifically binds *F. occidentalis* midguts and inhibits virus acquisition, and bovine serum albumin (BSA) in feeding solutions and another viral glycoprotein (HCMV gB,) did not inhibit TSWV acquisition by larval thrips (32).

G_N-S **reduced TSWV transmission when thrips were fed purified protein prior to AAP on TSWV-infected plant tissue.** To rule out the possibility that reduced virus acquisition in the presence of G_N-S is due to a direct interference of TSWV virions when mixed with G_N-S, thrips were fed on the G_N-S solution prior to an AAP on TSWV-infected *D. stramonium* leaves. Thrips were tested 2 to 3, 4 to 5, and 6 to 7 days post-pupal eclosion for TSWV transmission to determine if the G_N-S reduction in transmission continues over time in the adult insects. The incidence of transmitting insects fed TSWV alone was significantly different
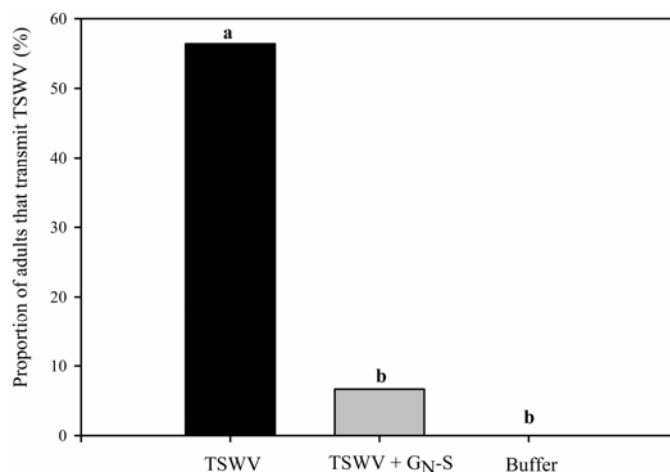

Fig. 1. Transmission of *Tomato spotted wilt virus* (TSWV) by *Frankliniella occidentalis* in a concomitant feeding experiment (experiment 1). Plants used for virus purification were harvested 2 weeks postinoculation and TSWV virions were isolated from infected *Datura stramonium* leaves using a modified and shortened version of the Gonsalves and Truijillo (7) procedure. Thrips were reared to adulthood on bean pods and given a 6-day inoculation access period on *D. stramonium* leaf disks. Individual leaf disks were assayed for TSWV infection by double-antibody sandwich enzyme-linked immuno-sorbent assay (DAS-ELISA) to determine the proportion of transmitting adults in the three thrips treatments. Bars headed with different letters are significantly different determined by Fisher's exact test ($P < 0.05$).

($P = 0.006$) when compared to those sequentially fed G_N-S and TSWV and at all times tested (Fig. 2). Approximately 50% of insects that were fed on the BSA-buffer solution transmitted TSWV up to the 5-day IAP. In contrast, less than 10% of the insects given the G_N-S solution prior to AAP on TSWV-infected leaves transmitted virus (Fig. 2). The TSWV-thrips midgut interaction was specific, as demonstrated by the inability of BSA to interfere with TSWV binding and transmission. These data indicate that inhibition of acquisition through pre-feeding on G_N-S effectively reduced TSWV transmission and corroborates the findings obtained in the concomitant feeding experiments. In treatments in which larval thrips were fed TSWV alone, the resulting infected adults had a reduced ability to transmit virus as they matured ($P = 0.048$). These results suggest that thrips transmission of TSWV decreases as the adult thrips age.

Virus infection status of transmitting and nontransmitting thrips. In this separate sequential AAP transmission experiment, virus titer was quantified in individual insects to determine if reduced TSWV-transmission by thrips was associated with low viral load in the insect. We determined that the proportion of
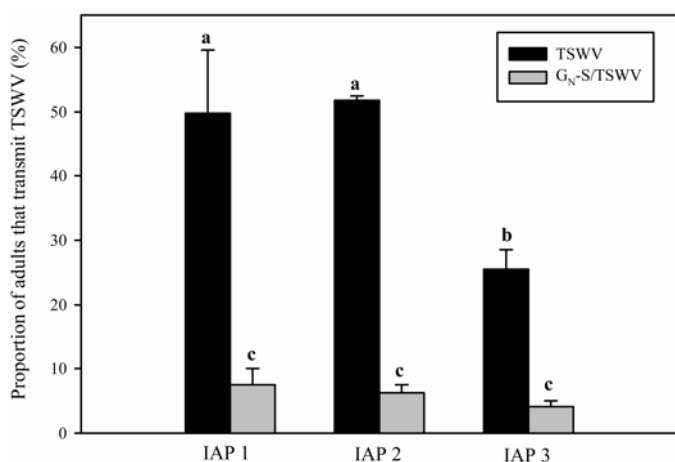
thrips infected with virus was reduced threefold when insects were preexposed to the G_N-S protein as compared to no exposure to protein ($P = 0.006$, Table 1), a finding which closely mirrored the proportion of leaf disks infected with the virus. In those insects that tested positive for TSWV, there was no apparent difference in the amount of virus replication between the two treatments ($P = 0.28$, Table 1), indicating that successful acquisition of TSWV in the presence of G_N-S resulted in virus replication in midgut tissues, infection of the salivary glands, and subsequent transmission. In addition, nontransmitting insects from the G_N-S/TSWV treatment did not contain detectable amounts of virus. These findings provide evidence that an initial reduction in virus acquisition in larval thrips, and subsequent infection of midgut tissues (represented by virus titer in adult thrips) are critical for preventing a transmission event (incidence of leaf disks infected).

Local lesion assay. To determine if G_N-S had a direct effect on virus infectivity of plants, G_N-S was mixed with purified TSWV and rub-inoculated onto local lesion-plant hosts. The number of local lesions per leaf were the same for the G_N-S + TSWV (average number of lesions per leaf = 21) and TSWV alone treatments (average number of lesions per leaf = 21). These results indicate that G_N-S did not affect the infectivity of purified virus, and support the hypothesis that the observed inhibition of thrips virus acquisition and the resulting reduced transmission are likely due to interference with the ability of TSWV glycoproteins to bind a host cell receptor(s) in the thrips midgut.

DISCUSSION

Our findings indicate that exogenous soluble viral glycoprotein can reduce transmission of a membrane bound virus by its insect vector in vivo. Because there is no continuous cell culture system for thrips, we examined the ability of G_N-S to inhibit TSWV infection of thrips via feeding experiments. Transmission assays showed that when thrips were fed G_N-S and purified TSWV concomitantly and when insects were first fed G_N-S and then placed on infected plants for acquisition of virus, TSWV transmission was reduced. Previous immunolabeling experiments revealed that G_N-S blocks TSWV acquisition at the first site of virus infection, the midgut epithelium (32). Collectively, these findings are consistent with G_N playing a critical role in TSWV infection of thrips, and it is likely that G_N-S binds molecules on the thrips midgut thereby preventing TSWV binding and entry. Our finding that G_N-S inhibits TSWV transmission is quite surprising because one might expect that although G_N-S reduces binding and acquisition, a small number of virions may bind the midgut and enter the epithelial cells. Subsequent replication and virus spread to surrounding cells could overcome this initial reduction in binding and therefore transmission proceeds at the usual frequency. We found that this was not the case, and therefore challenges our understanding of a propagative virus in its

Fig. 2. Transmission of *Tomato spotted wilt virus* (TSWV) by *Frankliniella occidentalis* in a sequential feeding experiment (experiment 2). Thrips were collected 0 to 24 h posteclosion from eggs and fed on solutions containing buffer or G_N-S (2h). Thrips were then placed on a bouquet of TSWV-infected *Datura stramonium* leaves for acquisition access (3 h). Thrips were reared to adulthood on bean pods and given a 2-day inoculation access period on *D. stramonium* leaf disks. For each insect, transmission was assayed three times after adult eclosion. Inoculation access periods (IAP 1, IAP 2, and IAP 3) were 2 to 3, 4 to 5, and 6 to 7 days after adult eclosion, respectively. Individual leaf disks were assayed for TSWV infection by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to determine the proportion of transmitting adults for each treatment. Bars represent means of three independent experiments with N = 104 (G_N-S followed by access to TSWV-infected plants) and N = 107 (buffer followed by access to TSWV-infected plants) leaf disks per treatment. Bars headed by different letters are significantly different at $P < 0.05$.

TABLE 1. Incidence and amount of *Tomato spotted wilt virus* (TSWV) infection in thrips[u] and leaf disks[v] exposed to thrips

Treatment[w]	Proportion of infected thrips	Virus titer in thrips[x]	Proportion of infected leaf disks	Amount of virus in leaf disks
Buffer/TSWV	68% (13/19) a[y]	1.69 ± 1.50 a[z]	75% (15/20) a	1.97 ± 0.17 a[z]
G_N-S/TSWV	19% (3/18) b	2.61 ± 0.86 a	20% (4/20) b	2.06 ± 0.31 a

[u] The amount of TSWV in individual thrips was quantified by real-time reverse transcriptase-polymerase chain reaction (PCR) using methods similar to those described in Boonham et al. (3).

[v] Individual leaf disks were assayed for TSWV infection by double-antibody sandwich enzyme-linked immunosorbent assay at OD_{405nm}.

[w] Treatments include thrips given a 2-h acquisition access period (AAP) on buffer solution and thrips given an AAP on buffer solution with G_N-S. Insects were then placed on a bouquet of TSWV-infected *Datura stramonium* leaves for 3 h. Thrips were reared to adulthood on healthy bean pods and after adult eclosion were placed on individual *D. stramonium* leaf disks for a 2-day inoculation access period (IAP).

[x] Virus titer is expressed as the abundance of TSWV nucleocapsid (N) RNA relative to thrips actin RNA using the inverse equation of Pfaffl (21): Relative abundance of $N = (E_{actin}{}^{Ct(actin)}/E_N{}^{Ct(N)}$; where E = PCR efficiency of a primer pair (actin or N) and Ct = threshold cycle of amplification.

[y] Column values followed by different letters are significantly different between treatments at $P < 0.05$.

[z] Values represent the average ± standard error for TSWV-positive samples (thrips or leaf disks).

vector. While similar binding inhibition has been documented for other viruses using tissue culture cells, we have conducted biological assays with live insect vectors to ask basic questions about enveloped virus transmission.

The importance of acquisition inhibition in reducing virus transmission is also supported by research with a nonenveloped plant-infecting virus, *Rice ragged stunt virus* (RRSV), which is transmitted by rice brown planthoppers (9). Insects given an AAP on viral spike protein prior to access on RRSV-infected plants showed a marked decrease in virus transmission compared to those fed infected plants alone. These results, taken in conjunction with the findings of the present study, support a model in which early binding events are critical to insect acquisition of both nonenveloped and enveloped viruses and that these events can be inhibited via exogenously applied viral attachment protein as a means of disrupting the transmission process. We have documented that a single G_N-S dose (0.1 nM) to thrips consistently reduced both TSWV binding of midgut cells (32) and transmission to plant cells (present study). In future experiments, we will examine the quantitative limits of using G_N-S to prevent transmission of TSWV by conducting dose-response experiments to determine the lowest effective dose of G_N-S required to break the TSWV transmission cycle. Saturation and competition binding experiments will be conducted to further characterize the affinity and specificity of G_N-S binding to a midgut molecule(s).

The ability of an adult thrips to transmit TSWV was dependent on its infection status. With the use of real-time RT-PCR to quantify TSWV-N transcripts on a per insect basis, we were able to determine that nontransmitting insects from the G_N-S/TSWV treatment did not contain detectable amounts of virus, a finding that corroborates the reduction in proportion of transmitting adults in the feeding experiments. Likewise, thrips that successfully transmitted the virus in either treatment (G_N-S/TSWV or TSWV alone) contained comparable amounts of virus, evidence that G_N-S did not affect virus replication. The "virus escapes" in thrips exposed to exogenous G_N-S may have resulted from a small proportion of thrips that did not acquire an effective dose of the glycoprotein to sufficiently reduce the amount of virus crossing the midgut barrier. Alternatively, it is possible that the virus receptor varies within the thrips population and G_N-S did not effectively block some of the receptors which would enable TSWV to enter the midgut of a proportion of the thrips population. In conjunction with the transmission studies, the real-time RT-PCR determination of virus titer suggests that it is critical to block initial acquisition because once the virus crosses the midgut barrier, replication and movement of the virus is unaffected by G_N-S.

The rate of virus replication in the midgut and the extent of virus migration from the midgut to the visceral muscle cells and the salivary glands are crucial factors in the determination of vector competence (19,21). Our finding that G_N inhibition of TSWV entry into the midgut also inhibits transmission is consistent with developmental studies revealing the significance of thrips ontogeny and virus spread to the salivary glands. Moritz et al. (19) proposed that the window for thrips acquisition is in the early larval stages because this is the only time during development when there is direct contact between the visceral muscles and the salivary glands, providing a pathway for virions to enter the salivary glands. In light of this hypothesis, an initial reduction in TSWV infection of midgut cells may prevent virus from reaching the muscle cells when they are in close contact with the salivary glands. Nagata et al. (21) also demonstrated the importance of salivary gland infection to TSWV transmission. They observed that thrips transmission correlated with the number of salivary glands infected, and insects with two salivary lobes infected were the best TSWV transmitters and insects with no salivary gland infection were unable to transmit TSWV. This is consistent with our real-time RT-PCR analysis of virus titer in insects that revealed nontransmitters fed G_N-S and virus did not contain detectable amounts of virus. This result concurs with the previous observation that G_N-S reduced TSWV infection in the midgut (32). It follows that G_N-S binding likely prevents infection of the midgut epithelial cells, which would subsequently result in poor or no infection of the salivary glands.

Our findings strongly support a model in which glycoproteins are critical for infection of midgut cells by enveloped viruses. Comparison of our findings with experiments showing the importance of G_N in the *La Crosse virus* (LAC)-mosquito interaction suggests that animal and plant infecting viruses in the family *Bunyaviridae* use similar mechanisms to infect arthropod vectors. Several parallels can be drawn between tospovirus and animal-infecting bunyavirus glycoprotein function. Consistent with our findings that G_N-S bound larval thrips midguts and inhibited virus transmission, scientists have found that LAC G_N binds mosquito cells in vitro (16). Observations of binding in dissected mosquito midguts suggest this is the initial site of LAC infection of mosquitoes.

Viruses have an intimate association with their host cells, creating an obstacle for the development of specific and durable virus control strategies. Identifying unique steps in the virus lifecycle is essential for developing specific control strategies. Because virus binding and entry are the first steps in the virus infection process, these early infection events are attractive candidates for the design of antiviral strategies. Our experiments show that using G_N-S to block thrips acquisition of TSWV in vivo may be a viable method of reducing virus transmission to crop plants. This strategy has been used effectively as a therapy for HIV; scientists identified a 36-amino-acid peptide derived from HIV-I gp41 (enfuvirtide) that disrupts the conformational changes required for fusion of viral and host membranes (5). This glycoprotein-derived peptide has been successfully deployed as an antiviral strategy (17). The importance of viral glycoproteins in infection of insect and mammalian hosts and our success in reducing TSWV transmission with G_N-S imply that therapeutic strategies like those used for HIV could be extended to insect-transmitted viruses of plants and humans. Ultimately, we hope to use this information to develop effective and specific control strategies for TSWV and thrips. Based on the ability of G_N-S to bind larval guts and inhibit TSWV transmission, the G_N protein is a good candidate for development of antiviral compounds.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Agresti, A. 1990. Categorical Data Analysis. John Wiley, New York, NY.
2. Bandla, M. D., Campbell, L. R., Ullman, D. E., and Sherwood, J. L. 1998. Interaction of Tomato spotted wilt tospovirus (TSWV) glycoproteins with a thrips midgut protein, a potential cellular receptor for TSWV. Phytopathology 88:98-104.
3. Boonham, N., Smith, P., Walsh, K., Tame, J., Morris, J., Spence, N., Bennison, J., and Barker, I. 2002. Detection of tomato spotted wilt virus (TSWV) in individual thrips using real time fluorescent RT-PCR (Taqman). J. Virol. Methods. 101:37-48.
4. Campbell, L. R., Robb, K. L., and Ullman, D. E. 2007. The complete tospovirus host list. Published online by Kansas State University. http://www.oznet.ksu.edu.er.lib.ksu.edu/tospovirus/hostlist.html.
5. Cooper, D. A., and Lange, J. M. A. 2004. Peptide inhibitors of virus–cell fusion: Enfuvirtide as a case study in clinical discovery and development. Lancet. Infect. Dis. 4:426-436.
6. Ebihara, H., Yoshimatsu, K., Ogino, M., Araki, K., Ami, Y., Kariwa, H., Takashima, I., Li, D., and Arikawa, J. 2000. Pathogenicity of *Hantaan virus* in newborn mice: Genetic reassortant study demonstrating that a single amino acid change in glycoprotein G1 is related to virulence. J. Virol. 74:9245-9255.
7. Gonsalves, D., and Trujillo, E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.

8. Griot, C., Pekosz, A., Lukac, D., Scherer, S. S., Stillmock, K., Schmeidler, D., Endres, M. J., Gonzalez-Scarano, F., and Nathanson, N. 1993. Polygenic control of neuroinvasiveness in California serogroup bunyaviruses. J. Virol. 67:3861-3867.

9. Guoying, Z., Xiongbin, L., and Huijuan, L. 1999. *Rice Ragged Stunt Oryzavirus*: Role of the viral spike protein in transmission by the insect vector. Ann. Appl. Biol. 135:573-578.

10. Hacker, J. K., and Hardy, J. L. 1997. Adsorptive endocytosis of California encephalitis virus into mosquito and mammalian cells: A role for G1. Virology 235:40-47.

11. Hunter, W. B., Hsu, H. T., and Lawson, R. H. 1995. A novel method for tospovirus acquisition by thrips. Phytopathology 85:480-483.

12. Janssen, R. S., Nathanson, N., Endres, M. J., and Gonzalez-Scarano, F. 1986. Virulence of La Crosse virus is under polygenic control. J. Virol. 59:1-7.

13. Jin, M., Park, J., Lee, S., Park, B., Shin, J., Song, K., Ahn, T., Hwang, S., Ahn, B., and Ahn, K. 2002. Hantaan virus enters cells by clathrin-dependent receptor-mediated endocytosis. Virology 294:60-69.

14. Koch, J., Liang, M., Queitsch, I., Kraus, A. A., and Bautz, E. K. F. 2003. Human recombinant neutralizing antibodies against Hantaan virus G2 protein. Virology 308:64-73.

15. Liang, M., Mahler, M., Koch, J., Ji, Y., Li, D., Schmaljohn, C., and Bautz, E. K. F. 2003. Generation of an HFRS patient-derived neutralizing recombinant antibody to Hantaan virus G1 protein and definition of the neutralizing domain. J. Med. Virol. 69:99-107.

16. Ludwig, G. V., Israel, B. A., Christensen, B. M., Yuill, T. M., and Schultz, K. T. 1991. Role of La Crosse virus glycoproteins in attachment of virus to host cells. Virology 181:564-571.

17. Matthews, T., Salgo, M., Greenberg, M., Chung, J., DeMasi, R., and Bolognesi, R. 2004. Enfuvirtide: The first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nat. Rev. Drug Discov. 3:215-225.

18. Mohamad, N. A. 1981. Isolation and characterization of subviral particles of tomato spotted wilt virus. J. Gen. Virol. 53:197-206.

19. Moritz, A., Kumm, S., and Mound, L. 2004. Tospovirus transmission depends on thrips ontogeny. Virus Res. 100:143-149.

20. Nagata, T., Nagata-Inoue, A. K., Prins, M., Goldbach, R., and Peters, D. 2000. Impeded thrips transmission of defective tomato spotted wilt virus isolates. Phytopathology 90:454-459.

21. Nagata, T., Inoue-Nagata, A. K., van Lent, J., Goldbach, R., and Peters, D. 2002. Factors determining vector competence and specificity for transmission of tomato spotted wilt virus. J. Gen. Virol. 83:663-671.

22. Pekosz, A., Griot, C., Nathanson, N., and Gonzalez-Scarano, F. 1995. Tropism of *Bunyaviruses*: Evidence for a G1 glycoprotein-mediated entry pathway common to the California serogroup. Virology 214:339-348.

23. Pekosz, A., Griot, C., Stillmock, K., Nathanson, N., and Gonzalez-Scarano, F. 1995. Protection from La Crosse virus encephalitis with recombinant glycoproteins: Role of neutralizing anti-G1 antibodies. J. Virol. 69:3475-3481.

24. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucl. Acids Res. 29:e45.

25. Resende, R., de, O., de Haan, P., de Avila, A. C., Kitajima, E. W., Kormelink, R., Goldbach, R., and Peters, D. 1991. Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. J. Gen. Virol. 72:2375-2383.

26. Schmaljohn, C. S., Chu, Y. K., Schmaljohn, A. L., and Dalrymple, J. M. 1990. Antigenic subunits of *Hantaan virus* expressed by baculovirus and vaccinia virus recombinants. J. Virol. 64:3162-3170.

27. Sin, S., McNulty, B. C., Kennedy, G. G., and Moyer, J. W. 2005. Viral genetic determinants for thrips transmission of tomato spotted wilt virus. P. Natl. Acad. Sci. USA 102:5168-5173.

28. Sundin, D., Beaty, B., Nathanson, N., and Gonzalez-Scarano, F. 1987. A G1 glycoprotein epitope of La Crosse virus: A determinant of infection of Aedes triseriatus. Science 235:591-593.

29. Tas, P. W. L., Boerjan, M. L., and Peters, D. 1977. The structural proteins of tomato spotted wilt virus. J. Gen. Virol. 36:267-279.

30. Ullman, D. E., Cho, J. J., Mau, R. F. L., Wescot, D. M., and Custer, D. M. 1992. A midgut barrier to tomato spotted wilt virus acquisition by adult western flower thrips. Phytopathology 82:1333-1342.

31. Ullman, D. E., German, T. L., Sherwood, J. L., Wescot, D. M., and Cantone, F. A. 1993. Tospovirus replication in insect vector cells: Immunocytochemical evidence that the nonstructural protein encoded by the S RNA of tomato spotted tospovirus is present in thrips vector cells. Phytopathology 83:456-463.

32. Whitfield, A. E., Ullman, D. E., and German, T. L. 2004. Expression and characterization of a soluble form of tomato spotted wilt virus glycoprotein G_N. J. Virol. 78:13197-13206.

33. Whitfield, A. E., Ullman, D. E., and German, T. L. 2005. Tospovirus-Thrips Interactions. Annu. Rev. Phytopathol. 43:459-489.

34. Wijkamp, I., and Peters, D. 1993. Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay. Phytopathology 83:986-991.